Physicochemical parameters influencing the formation of biofilms compared in mutant and wild-type cells of *Pseudomonas chlororaphis* O6

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Abstract Bacteria colonize surfaces as heterogeneous structures called biofilms. Intercellular communication using acyl homoserine lactones has been implicated in biofilm formation in some systems. Here, we investigate cell structure in biofilms and associated physiochemical properties of wild type and quorum sensing mutants of *Pseudomonas chlororaphis* O6 (*Pc*O6), a root-colonizing bacterium. The wild type strain generates multilayered biofilms under conditions where the quorum sensing mutant, deficient in the GacS sensor kinase, does not mature beyond a monolayer structure. However, this *gacS* mutant rapidly evolves to form a small colony variant (*gacS*-SCV) that again produces a multilayered biofilm structure although AHSL production is not restored to wild type level. Biofilms formed by the *gac*S-SCV (114 ± 12°) mutant were the most hydrophobic displaying a higher average ethylene glycol contact angle than those formed by the wild type (28 ± 7°) and *gac*S (18 ± 6°). Tapping mode atomic force microscopy revealed elongated cell structure in both of the mutant biofilm cells. Digital pulsed force mode adhesion mapping showed that the average adhesion followed the order *gacS* > *gacS*-SCV, wild-type. Certain of these *gac*S mutant cells displayed strong interactions of the AFM tip with cell boundaries, the role of which in biofilm formation is currently under investigation.

Keywords Biofilm hydrophobicity; biofilm structure; digital pulsed force mode; *Pseudomonas chlororaphis* O6

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Introduction

The organization of bacteria into multicellular communities called biofilms affords them significant advantages over their planktonic counterparts in terms of antibiotic resistance (Costerton *et al.*, 1999; Brooun *et al.*, 2000). In nature biofilms can be heterogeneous structures where macrocolonies are held together by extracellular materials forming classical mushroom-shaped pillars separated by voids that may serve to disseminate nutrients and remove waste material (Lawrence and Korber, 1991).

Biofilm formation requires gene regulation in the bacterial cell that is influenced by intercellular communication. Gram-negative bacteria may produce and sense intercellular signaling compounds that include acyl homoserine lactones (AHSLs) (Fuqua and Greenberg, 2002). In *P. aeruginosa*, an AHSL controls the production of the surfactant rhamnolipid that is required to maintain channel formation in layered biofilms (Davey *et al.*, 2003). In this work, we study a *gac*S mutant of the root-colonizing pseudomonad, *Pseudomonas chlororaphis* (*Pc*) *Pc*O6, which is impaired in its production of AHSLs (Spencer *et al.*, 2003). The synthesis of AHSLs is downstream of the interaction of the GacS sensor kinase with the regulatory GacA (Figure 1).

SEM studies of biofilms formed at a liquid–surface interface show that while wild type *Pc*O6 produces a thick and layered biofilm in rich medium, biofilms with the *gac*S mutant cells do not mature beyond the monolayer. The *gac*S mutant, however, rapidly reverts to a cell type, termed the *gac*S-SCV mutant, which again produces a thick layered

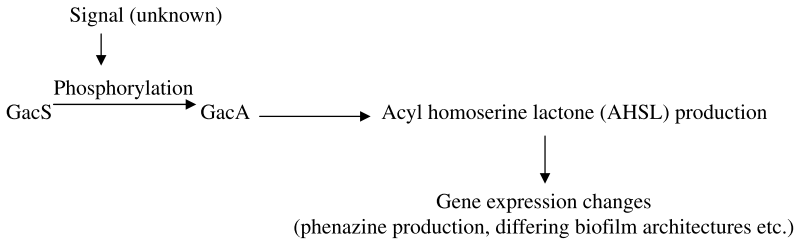

Figure 1 The relationship between the GacS/GacA signaling system and AHSL control of gene expression in *Pc*O6 (Spencer *et al.*, 2003)

biofilm (Figure 2). These biofilms were formed on polystyrene pegs suspended in inoculated King's medium B in a microtiter trough (MBEC™ Device, MBEC Biofilm Technologies, Edmonton, Alberta, Canada (Davies *et al.*, 2004, personal communication).

Here we explore the hypothesis that physicochemical parameters differing between the wild type and mutant isolates of *Pc*O6 may contribute to their varied ability to form biofilms growing at an air–agar surface interface. We used atomic force microscopy (AFM) to resolve the shape and size of the cells while growing as biofilms. The AFM can resolve structural features of live bacterial cells that have greater detail at the same magnification as SEM, but without extensive sample preparation (Palmer and Sternberg, 1999). We used digital pulsed force mode (DPFM) to generate an adhesion map simultaneously with the corresponding topographical image of the same sample (Zeiser *et al.*, 1997). To examine hydrophobicity differences between the biofilms of the three cell types sessile drop contact angle measurements were carried out using ethylene glycol as a polar probe liquid.

Materials and methods

The bacterial cells were grown from freezer stocks and plated directly onto Kings B medium (King *et al.*, 1954). They were incubated at 28°C for 4 days to generate confluent cultures. These biofilms were first analyzed using a Digital Instruments (Santa Barbara, CA, USA) Bioscope atomic force microscope in Tapping® mode at an amplitude set point that varied between 1 and 2 V. Next, the cantilever was brought into contact for adhesion mapping using the DPFM mode of operation (Witec Inc., Ulm, Germany) at a deflection set point of approximately 1 V. As a control, force curves were performed on plasma-cleaned glass between each cell type to ensure that the tip was not contaminated with material from the sample during scanning. All experiments were performed in duplicate and in ambient air. The cells were imaged over several scan sizes and at different scan angles to ensure that the features observed were not due to artifacts. Image analysis was performed using freeware WSxM (Nanotec Electronica, Spain) and Nanoscope software (Digital Instruments) for tapping mode images and Image Ctrl software (Witec Inc., Ulm, Germany) for DPFM images. Biofilm hydrophobicity was determined from sessile drop contact angle measurements with ethylene glycol (FW = 62.07, chemical formula $HOCH_2CH_2OH$) of 1 μL drop volume as the probe liquid, using a VCA Optima goniometer (AST Products, Bellerica MA, USA). Instantaneous contact angles were taken within 5 seconds of the drop touching the biofilm surface. The contact angle data were repeated for at least 5 to 10 samples over three separate studies for each of the cell types.

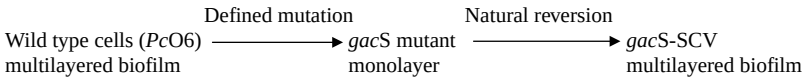

Figure 2 Schematic showing the generation of mutants in *Pseudomonas chlororaphis* O6 and their bioforming abilities

The contact angle values shown are instantaneous values taken within 5 seconds of the drop touching the biofilm surface, as the probe liquid is wicked away through the porous biofilm structure, contributing to changes in contact angles over time.

Results and discussion

AFM and DPFM (adhesion map) images (Figure 3) revealed elongated cell morphologies (Figure 3A) for the *gac*S-SCV and *gac*S mutants, a feature absent in the wild type. Cells of the wild type ranged from 1 to 2 μm in length, with no cells having a length greater than 3 μm (Figure 4). In the case of both *gacS* and *gacS*-SCV cells, elongated cells up to 5 μm in length were observed (Figure 4). As this unusual cell morphology is present for both *gac*S and *gac*S-SCV mutants, i.e. in cells that have differing potentials to form biofilms, it is unlikely that this cell morphology contributes to the differing biofilm architectures observed by SEM. Using AFM in the tapping mode a distinct central depression was observed on the cell surface. Although this may be an artifact of drying this is uncertain as drying of mature stationary phase biofilms has been shown to have a negligible effect on the cell surface properties (Auerbach *et al.*, 2000).

DPFM measurements (depicted in Figure 3D with corresponding topographical image in Figure 3C) revealed that averaged adhesive forces differed between the silicon nitride

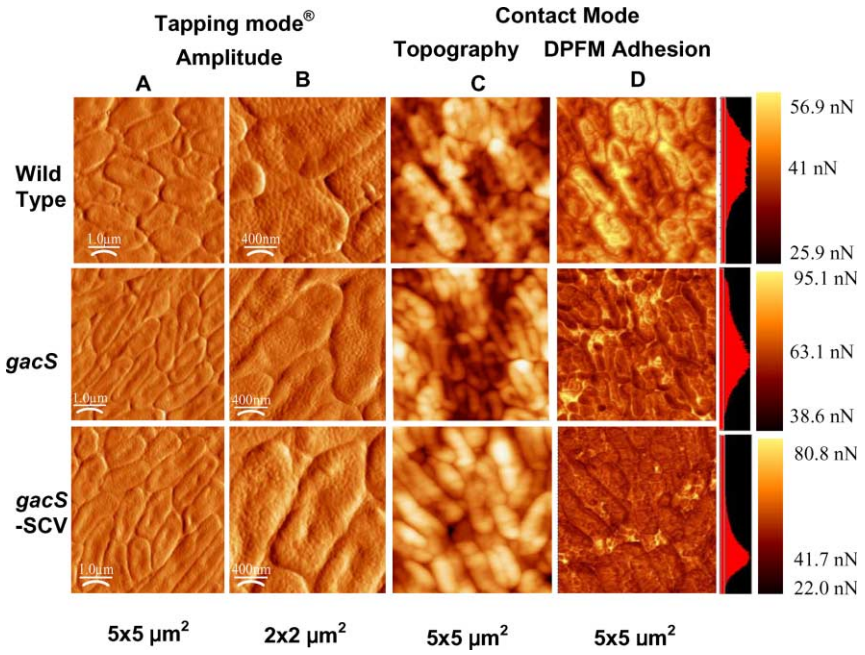

Figure 3 DPFM adhesion (D) images of *Pc*O6 wild type and mutants provide nanometer details of topography and adhesion properties of cells in each biofilm. (A) Topographical images taken using the AFM in the Tapping mode® at 5 × 5 μm^2 scan size clearly show elongated cells for the mutants absent in the wild type. (B) Second column of images representing magnified tapping mode images of cell topography at a 2 × 2 μm^2 scan size (C) Topography corresponding to the adhesion images scanned at a 5 × 5 μm^2 scan size in contact mode. A lighter color represents a higher surface feature and a darker color a lower one. (D) DPFM images of the same scan area as (C) showing adhesion varying from higher (lighter color) to lower (darker color). Adhesion histograms are also shown (extreme right) corresponding to these topographical images over the same scan area to provide an idea of how the adhesion varies spatially across the biofilms. Average adhesion values to the hydrophilic AFM tip followed the order *gac*S (63.1 nN) > *gac*S-SCV (41.7 nN), wild type (41 nN). Subscribers to the online version of *Water Science and Technology* can access the colour version of this figure from http://www.iwaponline.com/wst.

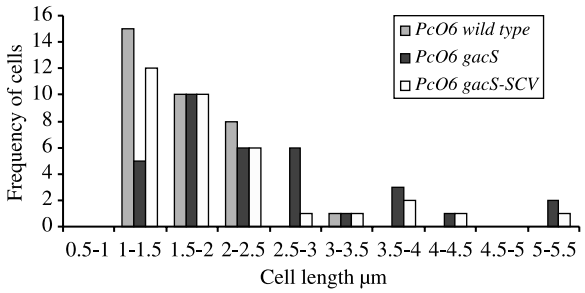

Figure 4 Histogram of the cell lengths of cells of *Pc*O6 wild type and mutants in biofilms on Kings medium B. Legend: WT = wild type; *gac*S = *gac*S mutant; *gac*S-SCV = small colony variant mutant. Measurements were made over three different fields of view using the Nanoscope software accompanying the AFM instrument. The wild type differs by the lack of cells beyond a 3 μm cell length. A total of 34 cells from three different fields of view were scored for each cell type

AFM tip and the cells. Average adhesive forces to the natively hydrophilic AFM tip followed the order *gacS* (63.1 nN) > *gacS*-SCV (41.7 nN), wild-type (41 nN) as represented by accompanying adhesion histograms. Force magnitude is represented for viewing by a color gradient ranging from light (low adhesion) to dark (representing strongest adhesion). Certain of the *gac*S mutant cells exhibited a greater interaction between the AFM tip and some of cell boundaries than the other two cell types (Figure 3D).

Assessment of the hydrophobicity of the cells in the biofilm surfaces showed highest average contact angle (114 ± 12°) with the *gacS*-SCV mutant. Values for the ethylene glycol drops were lower for the wild type (28 ± 7°) and the *gac*S mutant (18 ± 6°) cells (Figure 5A and B). Variance in contact angle values can be attributed to drying out of the biofilm during measurement and the "wicking away" or absorption of the probe liquid into the underlying biofilm surface and agar with time.

Conclusions

Morphological and physicochemical differences exist between biofilm cells of the wild type *Pc*O6 and its *gac*S and *gac*S-SCV mutants. One difference, the production of

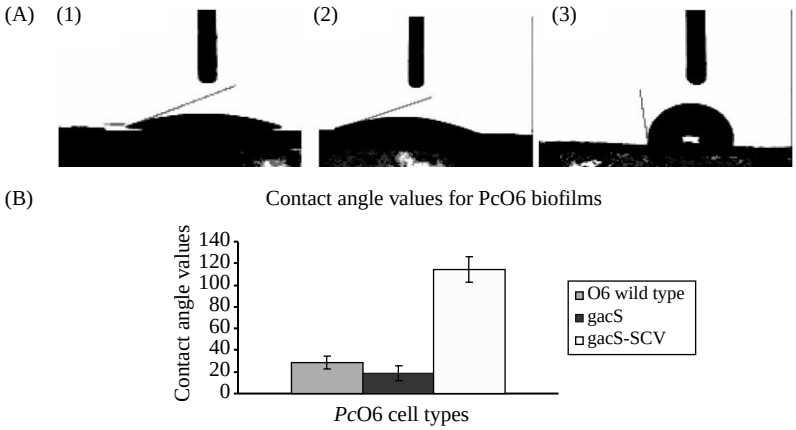

Figure 5 (A) Images of ethylene glycol sessile drops and their typical contact angles on biofilm surfaces of *Pc*O6 wild type and mutant cells. From left to right *Pc*O6 WT (1), *Pc*O6 *gac*S (2), *Pc*O6 *gac*S-SCV (3). (B) Bar chart of average contact angles with error bars. The *gacS*-SCV mutant has the largest contact angle (114 ± 12°), suggesting that it is the most hydrophobic of the three biofilms, followed by the wild type (28 ± 7°) and the *gac*S mutant (18 ± 6°). Contact angle values were considered for all trials, as the probe liquid wicks into the biofilm and underlying agar surface with time

elongated cells, exists with both the monolayer-forming *gac*S mutant and its natural revertant, the *gac*S-SCV mutant, which produces thicker biofilms. However, the *gac*S biofilm cells were more hydrophilic, as demonstrated by contact angle measurements, and showed stronger interaction between the tip and cell boundaries than the other two cell types. Whether or not this contributes to the monolayer biofilm architecture displayed by the *gac*S cell type is currently under investigation. Contact angle measurements showed the *gac*S-SCV cell type displayed a dramatically higher surface hydrophobicity than its parent *gac*S and wild-type cell types. This finding was consistent with a lower attraction to the natively hydrophilic AFM tip evinced by DPFM measurements. It is possible that this increased biofilm surface hydrophobicity could contribute to its multi-layered architecture. In ongoing work, we are employing hydrophobically-modified AFM tips to test our hypothesis that increased hydrophobic forces may contribute to multi-layered architecture of the *gac*S-SCV mutant cells. In summary, certain physiocochemical parameters varied between the *Pc*O6 wild type and mutant cells affecting their respective abilities to form biofilms.

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